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The objective of this proposal is to develop a clinically translatable strategy to restore the breast mound following mastectomy or lumpectomy such that patient quality of life and outcomes are markedly improved. Despite tremendous advances in surgical techniques and ancillary support devices, severe reconstructive limitations exist. A new rehabilitative strategy has emerged, namely the field of tissue engineering is attempting to overcome reconstructive limitations by developing strategies that permit patients to regrow their own breast mounds using autologous adipose tissue cells, specifically preadipocytes or adipose tissue precursor cells. This multidisciplinary application employs principles of bioengineering, synthetic polymer chemistry, and preadipocyte cell biology to develop an innovative biodegradable scaffold material capable of presenting bioactive molecules in a spatially and temporally controlled fashion leading to de novo adipose tissue formation.

We hypothesize that a novel material consisting of biodegradable poly(ethylene glycol) (PEG) hydrogels modified to present a critical cell adhesion molecule and degradation sequence can be seeded with preadipocytes to promote in vitro adipogenesis. The specific aims are to (1) develop a photopolymerizable and biodegradable PEG hydrogel derivatized with YIGSR and LGPA peptides and to bioactivity, and (2) demonstrate PA viability, proliferation, and differentiation within the PEG hydrogels.

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INTRODUCTION

The objective of this proposal is to develop a clinically translatable strategy to restore the breast mound following mastectomy or lumpectomy such that patient quality of life and outcomes are markedly improved. Despite tremendous advances in surgical techniques and ancillary support devices, severe reconstructive limitations exist. A new rehabilitative strategy has emerged, namely the field of tissue engineering is attempting to overcome reconstructive limitations by developing strategies that permit patients to regrow their own breast mounds using autologous adipose tissue cells, specifically preadipocytes or adipose tissue precursor cells. This multidisciplinary application employs principles of bioengineering, synthetic polymer chemistry, and preadipocyte cell biology to develop an innovative biodegradable scaffold material capable of presenting bioactive molecules in a spatially and temporally controlled fashion leading to de novo adipose tissue formation.

We hypothesize that a novel material consisting of biodegradable poly(ethylene glycol) (PEG) hydrogels modified to present a critical cell adhesion molecule and degradation sequence can be seeded with preadipocytes to promote in vitro adipogenesis. The specific aims are to (1) develop a photopolymerizable and biodegradable PEG hydrogel derivatized with YIGSR and LGPA peptides and to bioactivity, and (2) demonstrate PA viability, proliferation, and differentiation within the PEG hydrogels.

BODY

The original tasks stated that PEG hydrogels would be derivatized with fibroblast growth factor-10 (FGF-10). However, in the course of the research it was decided to concentrate first on derivatizing PEG hydrogels with a cell adhesion peptide sequence (YIGSR) and a degradation peptide sequence (LGPA). Hence, TASK 1 and 2 of the original Statement of Work was slightly modified (**bold text** below).

TASK 1: To develop a photopolymerizable and biodegradable PEG hydrogel derivatized with a **cell adhesion peptide and a degradation peptide sequence** by tethering **YIGSR and LGPA, respectively** to PEG using peptide linkers (Months 1-4):

a. Fabricate LGPA peptide sequence
b. Fabricate PEG-LGPA-diacrylate and characterize
c. Fabricate acryloyl-PEG-**YIGSR** and characterize
d. Photopolymerize to form hydrogels

TASK 2: To assess **PEG** bioactivity **and rheology**. (Months 5-6):

a. Measure PEG bioactivity and degradation.

b. **Measure rheological properties of PEG-YIGSR-LGPA hydrogels and compare with in vivo adipose tissue.**

TASK 3: To demonstrate PA viability, proliferation, and differentiation within the PEG hydrogels. (Months 7-12):

a. Seed hydrogels with culture rat preadipocytes (PAs)
b. Determine PA viability.
c. Determine PA proliferation and compare results with standard 2D proliferation on tissue culture plastic.
d. Determine the degree of PA differentiation using Oil Red O staining.

All tasks were successfully completed within the 1-year time frame and specific accomplishments and outcomes are listed below. The Appendices contain details of experiments via two submitted manuscripts.

KEY RESEARCH ACCOMPLISHMENTS

- Polymer chemistry was successful in generating PEG hydrogels derivatized with YIGSR (for cell adhesion) and LGPA (for degradation).
- Biological and rheological assessment of the hydrogels was completed.
- The project was successful and provides outstanding preliminary to garner more substantial and longer duration funding from National Institutes of Health and other funding sources.

REPORTABLE OUTCOMES

- Project was a portion of a graduate student's (Parul Patel) doctoral thesis. She successfully defended her PhD thesis and officially graduates with a Ph.D. in December 2005.
- Two manuscripts have been submitted to 1st tier, peer reviewed journals on this research (included in Appendices)
    - Rheological and Recovery Properties of Poly(ethylene glycol) Diacrylate Hydrogels and Human Adipose Tissue, Parul Natvar Patel, Connie Kathleen Smith, Charles W. Patrick, Jr., *Biomaterials*, submitted.
    - Poly(ethylene glycol) Hydrogel System Supports Preadipocyte Viability, Adhesion, and Proliferation, Parul Natvar Patel, Andrea S. Gobin, Jennifer L. West, Charles W. Patrick, Jr., *Tissue Engineering*, submitted.

CONCLUSIONS

The successful accomplishment of the tasks outlined above provide a strong foundation to proceed with the following:

- Incorporation of growth factors
- Conduct in vivo studies (i.e., transplant preadipocyte-loaded PEG hydrogels and generate adipose tissue in vivo).
- Apply for continued extramural funding

REFERENCES

(1) P.N. Patel, C.K. Smith, C.W. Patrick, Jr., Rheological and recovery properties of poly(ethylene glycol) diacrylate hydrogels and human adipose tissue, *Biomaterials*, submitted.

(2) P.N. Patel, A.S. Gobin, J.L. West, C.W. Patrick, Jr., Poly(ethylene glycol) hydrogel system supports preadipocyte viability, adhesion, and proliferation, *Tissue Engineering*, submitted.

APPENDICES

Draft copies of the 2 manuscripts submitted (refs 1 & 2).

FINAL DRAFT SUBMITTED TO *BIOMATERIALS*

Rheological and Recovery Properties of Poly(ethylene glycol) Diacrylate Hydrogels and Human Adipose Tissue

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Abstract

The viscosity and elastic and viscous moduli of poly(ethylene glycol) diacrylate (PEGDA) hydrogels (10% w/v, 6000 Da) and human abdominal adipose tissue are measured as a function of shear rate and frequency. Results indicate that both materials exhibit shear thinning and are viscoelastic in nature. Rheological tests suggest that the hydrogels become firmer as strain and frequency increase. Adipose tissue however begins to break apart at these higher strains and frequencies. This behavior is confirmed by measuring the complex modulus (which indicates firmness) of both materials as a function of strain. Recovery properties are also measured for each material as a function of deformation. While PEGDA hydrogels are able to recover up to 78% of their original height after 15% deformation, adipose tissue is not able to recover over the range of deformations tested. The frequencies and strains over which the tests are conducted are those physiologically experienced by the human body. The hydrogels are able to withstand this range of forces and, hence, are appropriate for use as a soft tissue filler material. Additionally, the hydrogels are determined to swell 38.1% ± 0.9% and this swelling is independent of surface area. The complex modulus of hydrogels of varying polymer concentrations is also measured as a function of strain to determine the effects of changing polymer content. These results indicate that the hydrogels become firmer due to the higher number of polymer chains and behave more elastically as polymer content increases.

Keywords: Adipose Tissue, Poly(ethylene glycol) Diacrylate, Hydrogels, Material Properties, Soft Tissue Filler

Introduction

Resections of various tumors of the head and neck, breast, and soft tissue, as well as trauma, congenital abnormalities, changes associated with aging, and a myriad of other medical conditions commonly result in contour deformities due to defects in the dermis and underlying subcutaneous adipose tissue. These can impair the aesthetic appearance, function, and psychological well-being of patients. Soft tissue restoration as a means of correcting these defects poses particular challenges for both the tissue engineer and the reconstructive surgeon. The ideal material for soft tissue restoration would be nonallergenic, nonpyrogenic, produce no disease states, look and feel natural, economical relative to standard of care, stable after implantation or injection, and easy to use in the operating room and outpatient setting[1]. For relatively small defects, the material should be versatile enough to be injected through small gauge needles, yet capable of being molded into a solid implant after injection[2]. One such material that meets all of these qualifications is PEGDA.

Although poly(ethylene glycol) and its derivatives have been extensively used in cell separations, coatings, and various pharmaceutical applications[3,4], a number of its material properties have not been determined to date[5]. This paper investigates the material properties of PEGDA as a scaffold material for adipose tissue engineering. In order to successfully design a scaffold to correct for soft tissue defects, one must characterize and understand the physical properties of the implantable material. To appropriately determine the amount of polymer solution to inject into a site, the amount

of swelling of the hydrogel must be known so that the defect is not overfilled. Swelling properties were found by determining the volume uptake of the hydrogels once polymerized and incubated in buffered saline. The rheological behavior of PEGDA hydrogels and adipose tissue was investigated over a range of forces that could be experienced on a daily basis using a fluids rheometer. These results are used to determine whether PEGDA is a suitable material to fill dermal and soft tissue defects. Steady and dynamic tests were conducted to determine the viscosity, elastic modulus (G'), and viscous modulus (G") at physiological conditions. Viscosity is described as "resistance to shear or flow," G' as the "solid-like" or elastic component of the gel, and G" as the "liquid-like" or viscous component. The complex modulus (G*), which indicates firmness of the material, was also determined for the samples. The elastic and viscous moduli make up the real and imaginary parts of the complex modulus as $G^* = G' + iG''$ [6].

Recovery properties were also determined using a Creep Indentation Apparatus[7-9] to measure the percent recovery of the material after exposure to varying deformations.

Materials and Methods

Synthesis of PEGDA

Hydrogel samples are prepared with PEGDA of molecular weight 6000 Da. Poly(ethylene glycol) is acrylated by dissolving 0.1 mmol/mL dry poly(ethylene glycol) (Fluka, Buchs, Switzerland), 0.4 mmol/mL acryloyl chloride (Sigma), and 0.2 mmol/mL triethylamine (Sigma) in anhydrous dichloromethane and stirring the resulting solution under argon overnight. Potassium carbonate (2 M, 0.8 mmol/mL, Sigma) is added to the

resulting PEGDA in a separatory funnel. Carbon dioxide is vented and the remaining emulsion is allowed to separate by gravity. The dense organic layer containing the PEGDA is separated from the aqueous phase and dried with anhydrous magnesium sulfate (Sigma). The solution is filtered to remove the magnesium sulfate and precipitated with diethyl ether. The solution is again filtered to separate PEGDA. PEGDA is dried under vacuum and stored frozen under argon.

Hydrogel formation for recovery and rheological tests

Hydrogels are formed by dissolving 0.1 g of PEGDA per mL of Hepes buffered saline (HBS, pH = 7.4) to obtain a 10% w/v solution. For the rheological tests on 5% and 20%, 0.05 and 0.2 g of PEGDA is dissolved per mL of HBS, respectively. The photoinitiator (60 mg/mL of Irgacure 184 in ethanol) is added at 27 mL per mL of polymer solution. The polymer solution is injected into a transparent, circular, glass mold (25 mm diameter, 0.5 mm height) and the solution in the mold is exposed to ultraviolet light (10 mW/cm^2) for 5 minutes, or until gellation is complete. The hydrogel is removed from the mold and placed into 5 mL of HBS and allowed to swell. The procedure is repeated to form each hydrogel.

Adipose tissue acquisition

Human adipose tissue from the abdomen of the female body is used for all tests. Tissue samples are incidental tissue remaining from reconstructive procedures. Tissue accrual adheres to Institutional and National Institutes of Health human subjects guidelines.

Donor patients are 45-65 years in age and tissue samples are sectioned from approximately 2-4 mm below the dermis.

Rheological testing

Rheological measurements employ a strain-controlled ARES 100FRT (Advanced Rheometric Expansion System - TA Instruments, New Castle, DE) rheometer using the 25 mm parallel plate geometry. The viscosity (η), elastic modulus (G'), and viscous modulus (G") are measured (n=6/test). Viscosity is measured with the motor in steady mode. Dynamic oscillatory modes are used to measure the elastic, viscous, and complex moduli. All tests are performed at 37.1 C over a range of strains (up to 25-40% pending location) and frequencies (up to 50 Hz) that the human body physiologically experiences on a daily basis [Personal communication with Dr. Tom Krouskop].

Recovery testing

Recovery properties are measured at varying strains using a Creep Indentation Apparatus[7-9]. Samples are exposed to a constant stress and the resulting strain is monitored (creep testing) to equilibrium. The deforming force is then removed and the recovery of the sample height at equilibrium is determined. The percent recovery is calculated based on the final deformation of the hydrogel.

Hydrogel formation for swelling tests

Varying amounts of 10% w/v polymer solution are placed in 48- and 96-well plates and exposed to ultraviolet light (10 mW/cm^2) for 5 minutes, or until gellation is complete.

The hydrogels are massed before and after swelling in HBS and the percent volume increase is calculated based on these measurements.

Results

One of the first properties determined during new material characterization is the relationship between viscosity and shear rate. A plot of viscosity (η) of PEGDA hydrogels and adipose tissue vs. shear rate (γ) (Figure 1) indicates that viscosity decreases for both materials as the shear rate increases. This behavior is known as shear thinning and is common to many polymeric materials.

Figure 2 plots the elastic modulus (G') and viscous modulus (G") of PEGDA hydrogels vs. frequency for strains ranging from 0.1 to 20%. The elastic modulus remains relatively constant over the strains and frequencies tested. In contrast, the viscous modulus increases as the strain increases over the range of frequencies tested. This indicates that the material is becoming firmer as the strain increases for the range of frequencies tested. The viscous modulus at 1% strain is not shown on the plot due to limitations in the resolution of the measured torque of the rheometer. The complex modulus (G*) of PEGDA hydrogels vs. strain for frequencies set at π, 5π, 10π, and 15π Hz is illustrated in Figure 3. The complex modulus stays relatively constant below 1% strain for all frequencies tested. This is known as the Hookean region and indicates that the material is linearly viscoelastic under these conditions [6]. Above 1% strain, the complex modulus becomes non-linear and the curves at constant strain begin to separate

more as frequency increases. This trend also verifies that the material is becoming firmer as the strain increases.

Figure 4 shows plots of elastic (G') and viscous moduli (G") of human adipose tissue vs. frequency for strains ranging from 0.1 to 20%. The viscous modulus is relatively constant over the strains and frequencies tested. As the strain increases however, the elastic modulus decreases. Figure 5 shows the complex modulus (G*) of adipose tissue vs. strain for frequencies set at π, 5π, 10π, and 15π Hz. The complex modulus is not significantly different for each of frequencies tested and decreases with increasing strain. For the adipose tissue, the complex modulus shows no linear viscoelastic region as was seen for the hydrogels. These results indicate that the sample is becoming less firm (i.e., breaking apart) as strain and frequency increase.

The percent recovery of hydrogels was measured when exposed to varying strains using a Creep Indentation Apparatus (Figure 6). As the hydrogel is exposed to greater deformations, the percent recovery to its original state is lessened (78% recovery after 15% deformation). Although hydrogels were not able to recover fully to their original state even after low deformation, these recovery properties are still superior to those of adipose tissue since the tissue was not able to recover after creep testing on the apparatus over the range of deformations tested.

The relationship between surface area and hydrogel swelling was also determined, as well as the effects of polymer concentration on the complex modulus for PEGDA.

Measurements of hydrogel volume increases as a function of surface area (Table 1) indicate that the amount of swelling is independent of surface area and is 38.1% ± 0.9% for 10% w/v PEGDA (MW 6000 Da) hydrogels. Comparison of the complex modulus of hydrogels at varying concentrations of polymer indicates that hydrogel firmness increases with increasing polymer concentration (Figure 7). This trend is attributed to the increased number of polymer chains in the hydrogel system.

Discussion

PEGDA hydrogels were found to exhibit rheological properties that are qualitatively superior to human adipose tissue, thus making it a suitable material for initial correction of soft tissue defects. Results of the tests to determine the relationship between viscosity of PEGDA hydrogels and shear rate (Figure 1) indicate that the samples exhibit shear thinning (viscosity decreases as shear rate increases). There are two possible reasons for this phenomenon. First, as the shear rate increases, some of the forces holding the gel structure together could weaken and the viscosity of the hydrogel decreases as a result. Alternatively, as the shear rate increases, the polymer chains could become partially elongated and line up with the shearing streamlines, which would cause a decrease in viscosity. To determine which of these events was causing the shear thinning of the material, dynamic tests were conducted to determine the viscoelastic behavior of the material.

Dynamic testing indicates that at low strains, the hydrogel is predominantly elastic over the range of frequencies tested. As the strain is increased, the viscous modulus increases while the elastic modulus remains constant, indicating that viscous elements are beginning to take effect. Plotting the complex modulus G* vs. strain (Figure 3) shows that at strains above 1%, the complex modulus increases as the frequency increases. These trends indicate that the hydrogel is becoming firmer as strain and frequency are increased. This can be attributed to the large number of entanglements within the system. As the hydrogel deforms, the polymer chains may begin to elongate but overlapping chain entanglement causes increased firmness of the hydrogel. The greater loss in elasticity at lower frequencies can also be attributed to the polymer chains having more time to slip.

It is important to also note the recovery behavior of the hydrogel in order to determine if the hydrogel has appropriate material properties for soft tissue defect correction. In other words, the material must be able to withstand the daily forces exposed to the site of correction. Exposing the PEGDA hydrogels to creep indentation followed by recovery testing showed that as the hydrogel is deformed, the ability to recover to its original state is lessened (Figure 6). Water loss resulting from creep indentation could cause a decrease in the sample height. Greater deformations of the hydrogel could also cause the polymer chains to break, which would result in the hydrogel being less able to recover to its original state. However, the hydrogel is still able to recover to 78% of its original state even after exposure to high strains, which makes it an acceptable material for initial implantation into the site defect.

After subjecting adipose tissue to oscillatory testing, it was found to be viscoelastic, much like many other biological tissues. The samples of adipose tissue tested exhibit shear thinning which is similar to the behavior of the hydrogels. As with the hydrogels, dynamic tests were also conducted to determine the reason for the decrease in viscosity with increasing shear rates. From the dynamic oscillatory testing (Figures 4 and 5), we found that the adipose tissue becomes less firm with increasing strains and frequencies. These results indicate that the adipose tissue is being broken apart with increasing forces on the material. Lipid lobules in adipose tissue are known to be susceptible to rupturing with minimal forces. This is also confirmed with the creep indentation/recovery testing. When placed on the creep indentation apparatus, the adipose tissue samples were not able to recover after even the smallest deformations were imposed.

The material properties of poly(ethylene glycol) hydrogels can be altered using varying concentrations of polymer solutions. Results from measuring the complex modulus of hydrogels of varying polymer concentrations indicate that the firmness of the hydrogel increases with increasing polymer concentrations (Figure 7). This trait is attributed to the increase in the number of polymer chains in the system, which causes an increase in the number of entanglements in the system. To alter material properties of the hydrogel, other variables can be manipulated as well. For example, varying the polymer chain length at a given concentration will affect the physical properties of the hydrogel. At a given concentration, increasing the polymer chain length results in fewer polymer chains in the system, which in turn causes a decrease the firmness of the hydrogel.

Incorporation of degradation sites and adhesion ligands into the polymer (common in tissue engineering applications) influences chain length as well, and can affect the material properties.

Conclusions

In order to better understand the behavior of PEGDA hydrogels in varying physiological conditions, we characterized the physical properties of the polymer and verified that they are qualitatively superior to those of adipose tissue. The viscosity and elastic, viscous, and complex modulus of the hydrogel and adipose tissue were measured as a function of varying shear rates, frequencies, and strains that the human body could experience on a daily basis. Additionally, the percent recovery of the hydrogels and adipose tissue after deformation was measured as a function of strain. Results indicate that the hydrogels are able to withstand the range of frequencies and strains that it could potentially face on a daily basis when incorporated into the human body. Adipose tissue alone, however, breaks apart when exposed to these same forces. Based on the results from these tests, we then determined that the polymer is an appropriate material for initial implantation into the soft tissue defect.

Acknowledgments

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Tables and Figures

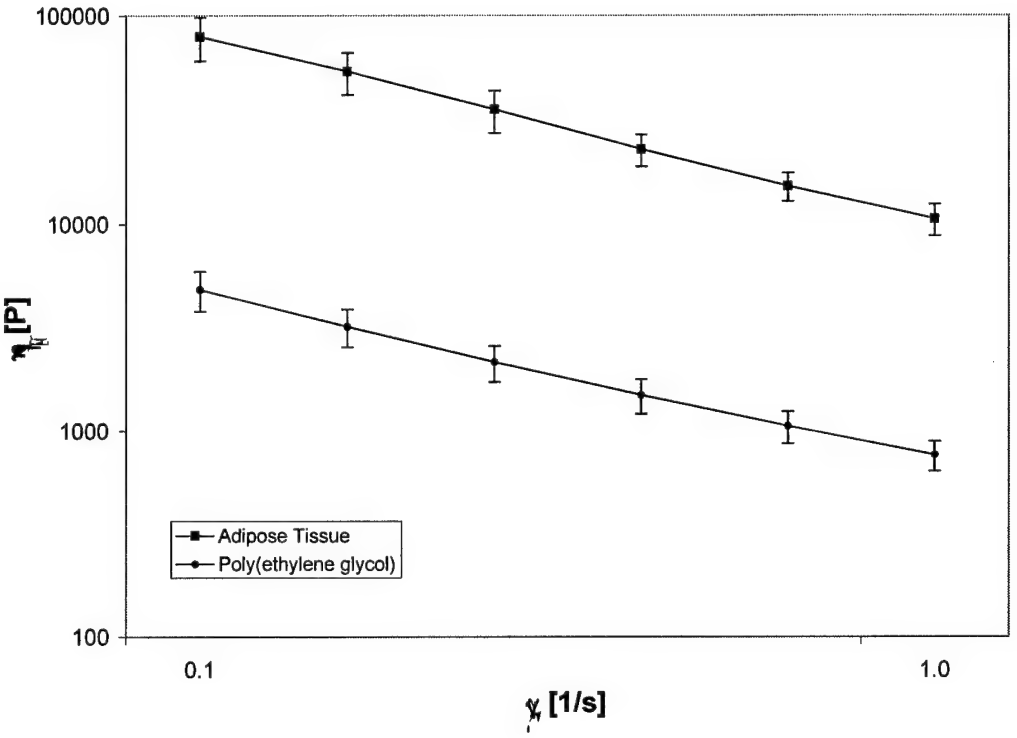


Figure 1. Viscosity vs. shear rate for poly(ethylene glycol) hydrogels and adipose tissue. Data are average viscosity (n = 10) ± standard error of the mean.

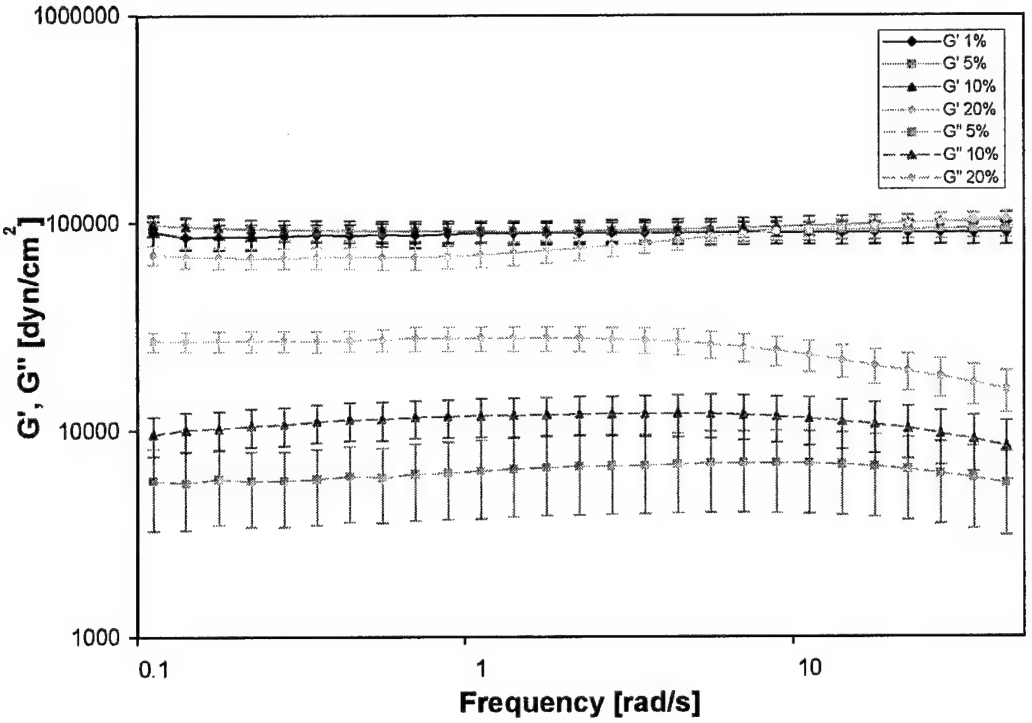


Figure 2. Elastic and viscous moduli of poly(ethylene glycol) hydrogel vs. frequency for strains set at 1%, 5%, 10%, and 20%. Data are average moduli (n=6) ± standard error of the mean.

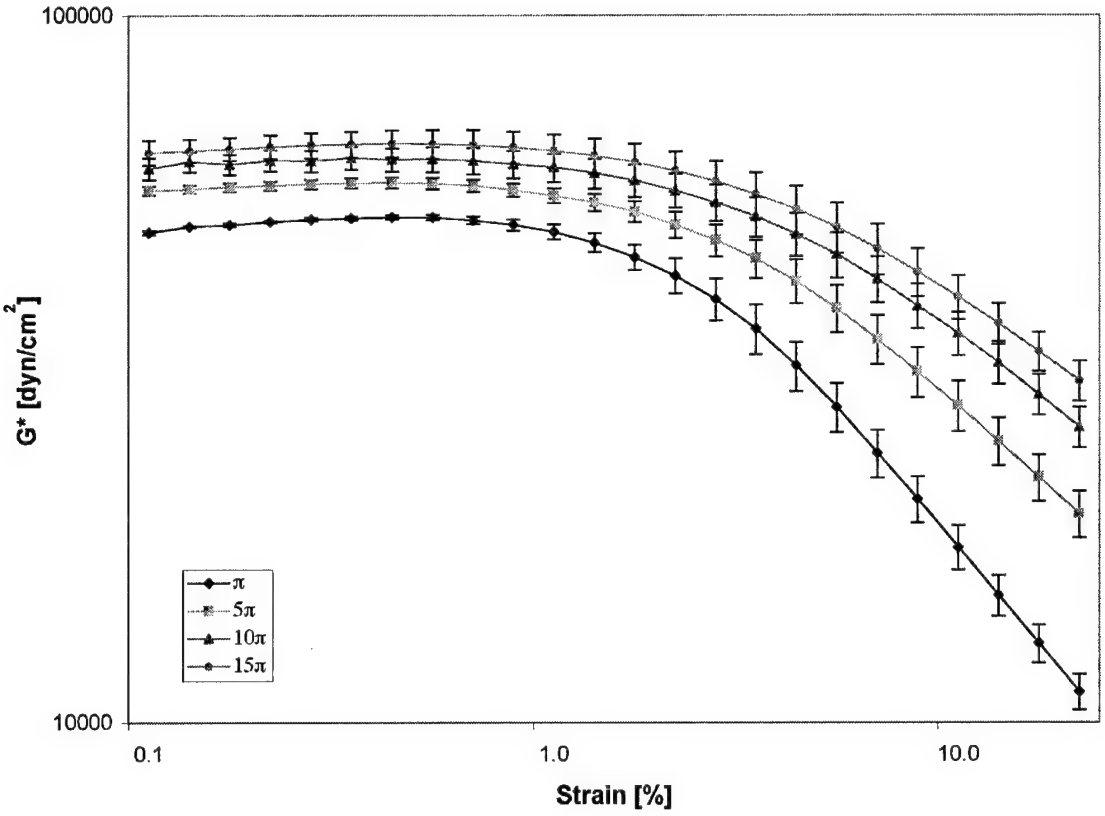


Figure 3. Complex modulus of poly(ethylene glycol) hydrogels vs. strain for frequencies set at π, 5π, 10π, 15π. Data are average moduli (n=6) ± standard error of the mean.

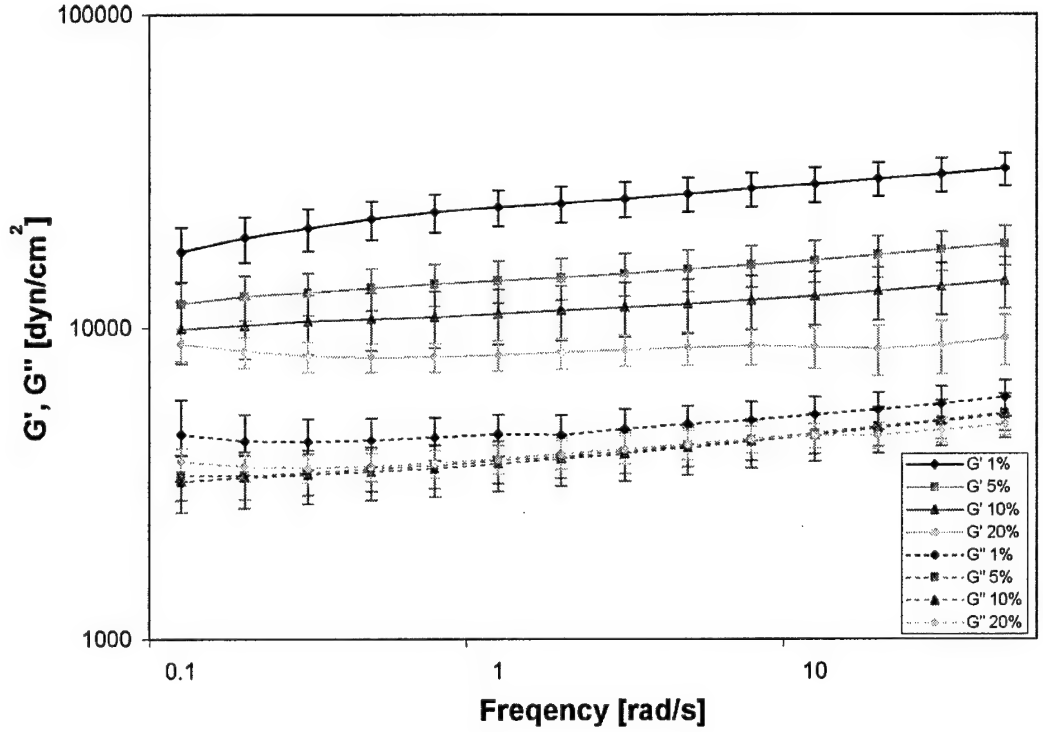
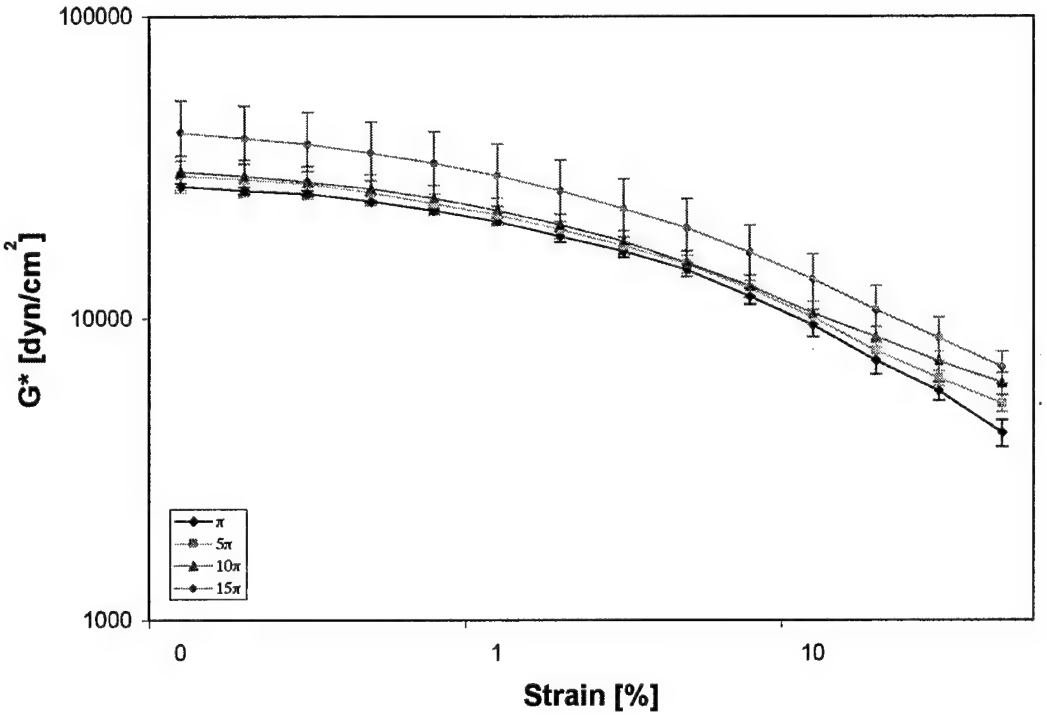


Figure 4. Elastic and viscous moduli of adipose tissue vs. frequency for strains set at 0.1%, 5%, 10%, and 20%. Data are average moduli (n=6) ± standard error of the mean.



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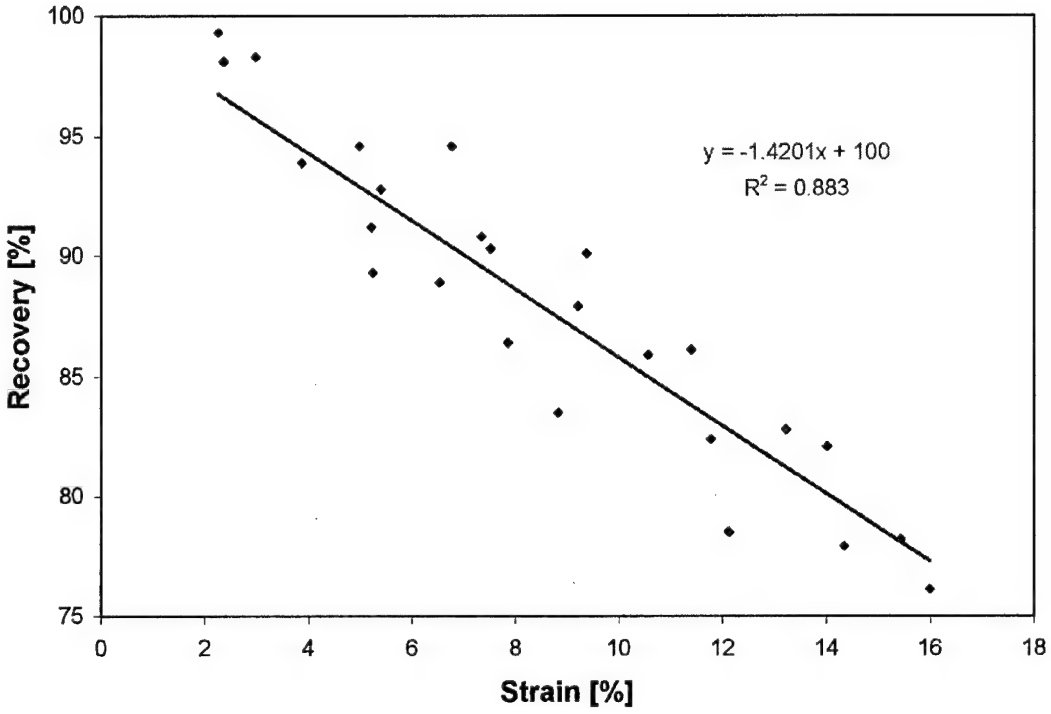

Figure 6. Percent recovery of poly(ethylene glycol) hydrogels from initial deformation as a function of strain.

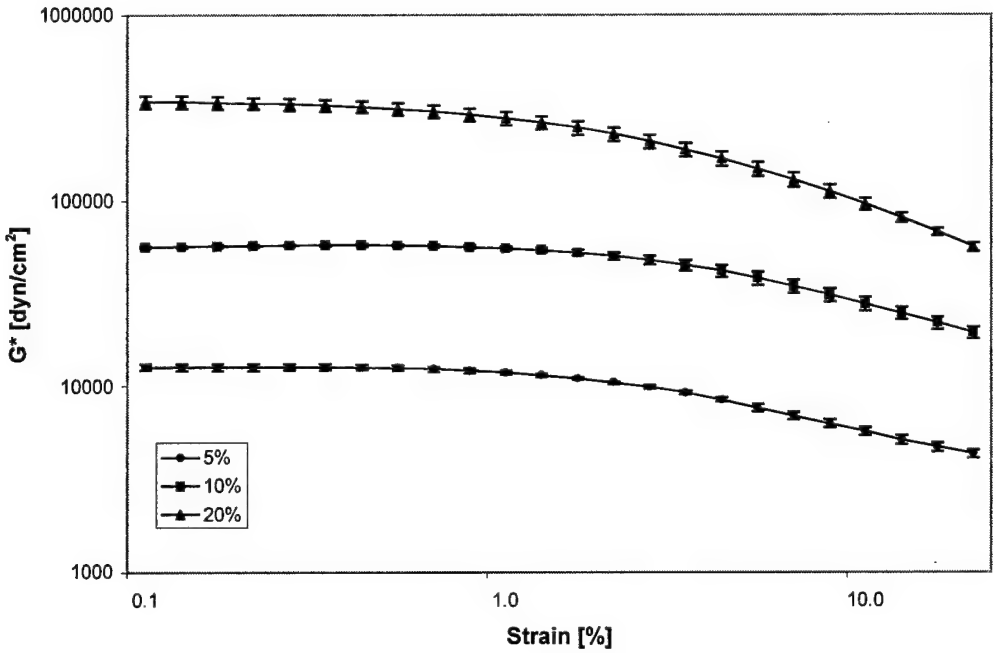

Figure 7. Complex modulus at frequency 5π vs. strain for poly(ethylene glycol) hydrogels of varying concentrations. Data are average moduli (n=6) ± standard error of the mean.

Geometry	Surface Area (cm^2)	Initial Volume (μL)	Final Volume (μL)	% Volume Increase	Average & SEM
96-well plate	0.95	50	72.1	44.1%	
		50	70.1	40.1%	44.1%
		50	74.1	48.1%	2.3%
	1.11	75	103.1	37.4%	
		75	109.1	45.5%	39.2%
		75	101.1	34.8%	3.2%
	1.27	100	144.1	44.1%	
		100	134.1	34.1%	37.4%
		100	134.1	34.1%	3.3%
	1.58	150	208.2	38.8%	
		150	216.2	44.1%	39.7%
		150	204.2	36.1%	2.4%
48-well plate	2.11	150	214.2	42.8%	
		150	206.2	37.4%	39.2%
		150	206.2	37.4%	1.8%
	2.32	200	272.2	36.1%	
		200	283.2	41.6%	38.3%
		200	274.2	37.1%	1.7%
	2.52	250	336.3	34.5%	
		250	334.3	33.7%	33.7%
		250	332.2	32.9%	0.5%
	2.73	300	406.3	35.4%	
		300	396.3	32.1%	33.3%
		300	397.3	32.4%	1.1%
Average Swelling					***38.1%***
SEM					***0.9%***

Table 1. Results of hydrogel swelling experiment.

FINAL DRAFT SUBMITTED TO *TISSUE ENGINEERING*

Poly(ethylene glycol) Hydrogel System Supports Preadipocyte Viability, Adhesion, and Proliferation

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Abstract

The ultimate goal of this research is to develop an injectable cell-scaffold system capable of permitting adipogenesis to abrogate soft tissue deficiencies resulting from trauma, tumor resection, and congenital abnormalities. The present work compares the efficacy of photopolymerizable poly(ethylene glycol) and specific derivatives as a scaffold for preadipocyte (adipocyte precursor cell) viability, adhesion, and proliferation. Four variations of a poly(ethylene glycol) scaffold are prepared and examined. The first scaffold consists of poly(ethylene glycol) diacrylate that is not susceptible to hydrolysis or enzymatic degradation. Preadipocyte death is observed over one week in this hydrogel configuration. Adhesion sites, specifically the laminin binding peptide sequence YIGSR, were incorporated into the second scaffold to promote cellular adhesion as a prerequisite for preadipocyte proliferation. Preadipocytes remain viable in this scaffold system, but do not proliferate in this nondegradable hydrogel. The third scaffold system studied consists of poly(ethylene glycol) modified with the peptide sequence LGPA to permit polymer degradation by cell-secreted collagenase. No adhesion peptide is incorporated into this scaffold system. Cellular proliferation is initially observed, followed by cell death. The previous three scaffold configurations do not permit preadipocyte adhesion *and* proliferation. In contrast, the fourth system studied, poly(ethylene glycol) system modified to incorporate both LGPA and YIGSR, permits preadipocyte adherence and proliferation subsequent to polymer degradation. Our results indicate that a scaffold system containing specific degradation sites and cell adhesion ligands permits cells to adhere and proliferate, thus providing a potential cell-scaffold system for adipogenesis.

Introduction

Over a million reconstructive, correctional, and cosmetic procedures are performed every year to repair dermal defects. Although many natural, synthetic, and hybrid materials are currently used clinically to correct contour deficiencies, none have proven to be the ideal material for soft tissue repair. Many of these materials possess severe limitations including, but not limited to, unpredictable outcome, fibrous capsular contraction, allergic reaction, suboptimal mechanical properties, distortion, migration, and long-term resorption.[1] The large number of soft tissue surgeries and the inadequacies of contemporary standard of care to correct soft tissue defects impact the national healthcare system. Moreover, patients are demanding more optimum outcomes and desire to use autologous cells.

Various adipose tissue engineering strategies are currently being investigated as a means to repair soft tissue defects. Scaffold materials for tissue engineering applications must be able to mechanically support and guide tissue formation. Materials must also be biocompatible, biodegradable, easily processed,[2,3] resistant to mechanical strain, and easily shaped to the surgeon's specifications.[4] In addition, materials must permit variability in shape and volume in order to personalize the scaffold to meet the patient's specific contour and volume needs. Ideally, the scaffold material should recapitulate the endogenous extracellular matrix in structure, support, and function.

This report discusses the use of photopolymerizable poly(ethylene glycol) hydrogels as scaffolds for adipose tissue engineering applications. Poly(ethylene glycol) hydrogels are

viscoelastic, three-dimensional polymeric structures that contain a significant volume fraction of water, usually >90%, and are held together primarily by covalent cross-linking. Photopolymerization of the hydrogels involves exposing the precursor polymer solution to an ultraviolet light source and allowing networks to form via free radical polymerization.[5] Photopolymerizable hydrogels are used extensively in medicine for biomedical applications[6] due to its ability to be crosslinked quickly *in situ*. Poly(ethylene glycol) or its derivatives have already been investigated as potential scaffold materials for bone[7] and cartilage tissue engineering,[8-10] as well as used in wound healing[2] and for the treatment of enzyme deficiencies.[11]

The present *in vitro* work investigates the use of diacrylated poly(ethylene glycol), a photopolymerizable hydrogel, as a scaffold for adipose tissue engineering. Poly(ethylene glycol) was chosen for this application due to its inherent biocompatibility and ability to permit tailored chemical modification, namely the addition of active cell adhesion and degradation sites.[12] Hydrogel systems made of nondegradable and degradable poly(ethylene glycol) with and without cell-adhesion ligands are studied in this report to evaluate preadipocyte viability, adhesion, and proliferation within each scaffold type.

Methods

Preadipocyte Isolation and Culture

Preadipocytes are isolated from epididymal fat pads of male Lewis rats (250g, Harlan) via enzymatic digestion and sequential filtering as previously described.[13] Briefly, rats are euthanized by CO_2 asphyxiation and the prepared harvest site is sterilized with

alcohol. Epididymal adipose tissue is aseptically harvested postmortum and placed in 4 C phosphate buffer solution (PBS) supplemented with 500 U/mL penicillin and 500 μg/mL streptomycin (Gibco). Harvested adipose tissue is finely minced and enzymatically digested in Ca^{+2}/Mg^{+2}-free PBS supplemented with 2% (w/v) type I collagenase (Sigma Chemical Co., St. Louis, MO) and 5% (w/v) bovine serum albumin for 20 minutes at 37 C on a shaker. The digested tissue is sequentially filtered through 250 μm and 40 μm nylon meshes to separate undigested debris and capillary fragments from preadipocytes. The filtered cell suspension is centrifuged for 5 minutes at 4 C, 200 g. The resulting pellet of preadipocytes is then plated at one-third confluency onto plastic culture flasks and preadipocytes are cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ mL penicillin, and 100 μg/mL streptomycin. The preadipocytes are supplied with fresh DMEM every other day. Preadipocytes are used prior to confluency since contact inhibition initiates adipocyte differentiation and ceases preadipocyte proliferation.[14-17] A cell concentration of 1 X 10^6 primary preadipocytes/mL is used in the following studies.

Synthesis of Poly(ethylene glycol) Diacrylate

Hydrogel samples are prepared with poly(ethylene glycol) diacrylate of molecular weight 6000 Da. Poly(ethylene glycol) is acrylated by dissolving 0.1 mmol/mL dry poly(ethylene glycol) (Fluka, Buchs, Switzerland), 0.4 mmol/mL acryloyl chloride (Sigma), and 0.2 mmol/mL triethylamine (Sigma) in anhydrous dichloromethane and stirring the resulting solution under argon overnight. Potassium carbonate (2 M, 0.8 mmol/mL, Sigma) is added to the resulting poly(ethylene glycol) diacrylate in a

separatory funnel. Carbon dioxide is vented and the remaining emulsion is allowed to separate by gravity. The dense organic layer containing the poly(ethylene glycol) diacrylate is separated from the aqueous phase and dried with anhydrous magnesium sulfate (Sigma). The solution is filtered to remove the magnesium sulfate and precipitated with diethyl ether. The solution is again filtered to separate poly(ethylene glycol) diacrylate. Poly(ethylene glycol) diacrylate is dried under vacuum and stored frozen under argon.

Preparation of Diacrylated Poly(ethylene glycol) Derivatives Containing Degradable Sequences

Preadipocytes have been shown to secrete collagenase to remodel its remodel its natural environment. Thus, a collagenase-sensitive polymer was prepared. Degradable poly(ethylene glycol) is synthesized by modifying acryloyl-poly(ethylene glycol)-N-hydroxysuccinimide (acrl-PEG-NHS, 3400 Da, Shearwater Polymers, Huntsville, AL) with the collagenase-sensitive peptide sequence glycine-glycine-leucine-glycine-proline-alanine-glycine-glycine-lysine (GGLGPAGGK) in a 2:1 polymer:peptide molar ratio. The polymer-peptide mixture is then dissolved in 50 mM sodium bicarbonate (pH 8.5) and allowed to react for 2 hours. The solution is filter sterilized, lyophilized, and stored frozen under argon. The resulting poly(ethylene glycol) derivative is a block copolymer, ABA, where A is poly(ethylene glycol) and B is the collagenase-sensitive peptide. The poly(ethylene glycol) derivative is terminated with acrylated groups for photocrosslinking (Figure 1a). An *in vitro* degradation assay is used to validate the specific degradation of this polymer.

Preparation of Monoacrylated Poly(ethylene glycol) Derivatives Containing Cell-Adhesion Ligands

Preadipocyte adhesion sites can also be coupled to poly(ethylene glycol) using the peptide sequence tyrosine-isoleucine-glycine-serine-arginine (YIGSR) (Figure 1b). YIGSR is one of many cell-binding peptides found on laminin-1. Wu and Patrick (2003) have shown that preadipocytes bind preferentially to laminin-1 and that cell adhesion and migration on laminin-1 is mediated by the $\alpha_1\beta_1$ integrin.[18] Acrl-PEG-NHS is combined with YIGSR in a 1:1 polymer:peptide molar ratio and then dissolved in 50 mM sodium bicarbonate (pH 8.5) and allowed to react for 2 hours. The solution is filter sterilized, lyophilized, and stored frozen under argon.

Photoinitiator Cytocompatibility with Preadipocytes

Various photoinitiators can be used to polymerize the poly(ethylene glycol) hydrogels. The ideal photoinitiator for the system however, is one that is cytocompatible with the cell type used. For example, Irgacure 184 is known to be cytocompatible with fibroblasts,[9] while Irgacure 651 is cytocompatible with endothelial cells[3] and Darocur 2959 with chondrocytes.[9] These three photoinitiators are tested for cytocompatibility with preadipocytes. Primary preadipocytes are seeded at 40,000 cells per well of 12-well plates in DMEM with serum. Three concentrations of each photoinitiator, varying from 0.025% (w/w) to 0.1% (w/w), are added to each plate. Control wells contained no photoinitiating system. Number of viable cells is recorded after two days using trypan blue exclusion and a Coulter cell counter (n=3/test condition).

Hydrogel Formation for Adhesion Study

To verify preadipocyte adhesion to the YIGSR ligands in the scaffold, poly(ethylene glycol) diacrylate hydrogels are prepared with and without the cell-adhesion peptide YIGSR. Poly(ethylene glycol) diacrylate is dissolved in Hepes buffered saline (pH 7.4) to achieve a 10% w/v solution. For the system containing adhesion sites, poly(ethylene glycol) coupled with YIGSR ligands is also added to achieve a 2.8 μmol per mL of polymer solution. Irgacure 184 (60 mg/mL in ethanol) is added to each system such that the photoinitiator concentration is 27 μL per mL of polymer solution. Results from the cytocompatibility study described above showed that Irgacure 184 is non-toxic to preadipocytes with the concentration employed. The polymer solution is placed in a 48-well plate at 200 μL/ well and exposed to ultraviolet light (10 mW/cm^2 at 365 nm) for 5 minutes. Hydrogels are incubated in DMEM with serum and allowed to swell at 37 C, 5% CO_2.

Adhesion Study

A solution of preadipocytes in DMEM is placed on the top surface of each system of hydrogels formed for this study. The hydrogel-cell system is then incubated for 3 hours at 37 C, 5% CO_2. The hydrogels are removed from incubation and each sample is gently washed twice with sterile buffered saline to remove any free cells. The hydrogels are then viewed for preadipocyte attachment using a Nikon Eclipse E600 upright microscope and 20x objective. Eight images are taken per system using a Hamamatsu EB-CCD camera (C7190, Nikon) and MetaMorph software (Universal Imaging, Downington, PA).

The number of cells attached per square area of each hydrogel system is then determined to assess the adhesive functionality of the YIGSR ligands used.

Hydrogel Formation for Viability and Proliferation Studies

Four configurations of scaffold systems are studied: (1) nondegradable hydrogel, (2) nondegradable hydrogel incorporated with cell-adhesion peptides, (3) degradable hydrogel, and (4) degradable hydrogel incorporated with cell-adhesion peptides. For the first two configurations studied, poly(ethylene glycol) diacrylate is dissolved in Hepes buffered saline (pH 7.4) at 0.2 g/mL. For the third and fourth configurations, poly(ethylene glycol) containing degradation sites is dissolved in Hepes buffered saline at 0.2 g/mL. A volume of solution of phenyl red-free DMEM and preadipocytes (1 X 10^6 cells/mL) is added equivalent to that of buffered saline added, such that the final polymer concentration is 10% w/v. In hydrogel configurations (2) and (4), poly(ethylene glycol) coupled with YIGSR ligands is also added to achieve a 2.8 μmol per mL of cell-polymer solution. The photoinitiator, Irgacure 184 (60 mg/mL in ethanol), is added to each system such that the photoinitiator concentration is 27 μL per mL of cell-polymer solution. The cell-polymer solution is placed in a 96-well plate at 100 μL/ well and exposed to ultraviolet light (10 mW/cm^2 at 365 nm) for 5 minutes. Hydrogels are incubated in DMEM with serum at 37 C, 5% CO_2.

Pico Green Double-Stranded DNA Assay

Hydrogel samples are analyzed in triplicate for DNA content at 0, 2, 4, and 6 days to assess cell proliferation. Samples described above are digested at each time point in 250

μL of 0.5 N sodium hydroxide and neutralized, and the DNA content in the gels is determined using a pico green double-stranded DNA assay (P-7581, Molecular Probes). This assay measures the amount of DNA in each sample at the time of digestion and is related to the cell proliferation within each hydrogel. Poly(ethylene glycol) diacrylate hydrogels containing no cells are used as the negative control. Hydrogels containing known amounts of calf thymus DNA (D-4522, Sigma) are used as standards to determine the DNA content of the sample gels. Samples are analyzed according to the manufacturer's instructions using a Bio-Rad fluorometer (excitation ~ 480 nm, emission ~ 520 nm).

Live/Dead Viability Stain

A two-color fluorescence viability assay (L-3224, Molecular Probes) is used to demonstrate the viability of preadipocytes in all four described hydrogel configurations six days subsequent to polymerization. Hydrogels with cells are incubated for 45 minutes in a combined 2mM calcein AM and 2 mM ethidium homodimer solution in sterile phosphate buffered saline. The calcein AM permeates live cells and produces a bright green fluorescence (ex ~495 nm/em ~515 nm), while the ethidium homodimer penetrates cells with injured membranes and produces a red fluorescence (ex ~495 nm/em ~635 nm).[19-20] Samples are viewed using a Retiga EXi FAST Cooled Mono 12-bit camera (QImaging, Burnaby, B.C. Canada) and IPLab software (Scanalytics, Inc., Fairfax, VA) for image analysis. The number of live and dead/dying cells is recorded for five planes in each of three samples for each hydrogel configuration and the percent live cells is then calculated.

Statistical Analysis

When appropriate, the means of data measurements are presented. Error bars are calculated as the standard error of the mean. Statistical significance is defined as $p \leq 0.05$ using a two-tailed, two-sample Student's t-Test assuming unequal variance.

Results

Hydrogel polymerizations for each of the configurations studied were successful and reproducible. Preadipocytes were found to be cytocompatible with Irgacure 184 and this photoinitiator was used during the gellation process. The results from the adhesion, proliferation, and viability studies are discussed below.

Photoinitiator Cytocompatibility with Preadipocytes

Figure 2 illustrates that even minimal amounts of Irgacure 651 caused significant preadipocyte death over the control group. The cytoincompatibility of Irgacure 651 with preadipocytes makes this photoinitiator a poor choice for use in crosslinking our polymer for preadipocyte seeding. Results show that both Darocur 2959 and Irgacure 184 are cytocompatible with preadipocytes for all concentrations tested. While Darocur 2959 does not affect cell viability, the relatively lengthy crosslinking time (>15 minutes) makes this photoinitiator an unsuitable choice for use in the clinical setting. However, the crosslinking time with Irgacure 184 is reasonably short (<5 minutes), which makes this photoinitiator an adequate photoinitiating system for preadipocyte seeding and

polymer crosslinking. For these reasons, Irgacure 184 was chosen to use as the crosslinking agent for all subsequent studies.

Adhesion Study

Results indicate that the YIGSR binding peptide used in these studies are functional for preadipocyte adhesion. Preadipocytes did not adhere to the surface of the hydrogels without the adhesion ligands. Cells were however able to attach to the surface of the hydrogels containing YIGSR, with the surface concentration calculated to be 107 cells/mm^2 (Figure 3). Thus, the adhesive functionality of the YIGSR is proven for preadipocyte attachment to this peptide sequence.

Pico Green Double-Stranded DNA Assay

Figure 4 compares the DNA content in each hydrogel configuration at varying time points. DNA content in both of the nondegradable hydrogel configurations does not increase over the experimental time course. These results suggest that preadipocyte proliferation does not occur in these two polymer configurations. In contrast, the DNA content in the degradable hydrogels (without YIGSR) increases initially and then decreases to initial levels. Only DNA content at day 4 is statistically different ($p = 0.016$) when compared to day 0. Unlike the previous three polymer configurations, the DNA content in degradable gels with cell adhesion sites increases four-to-five-fold over the experimental time course. DNA content at days 4 and 6 are statistically different ($p = 0.012$ and 0.006, respectively) when compared to day 0.

Live/Dead Viability Stain

Results indicate that as many as 90% of the cells in the hydrogels containing cell-adhesion ligands, configurations (2) and (4), remained viable for the duration of the experiment. Viability within the hydrogel configurations without YIGSR ligands was approximately 60% for the degradable scaffold and less than 10% in the nondegradable scaffold (Figure 5).

Discussion

Contemporary research includes the use of scaffolds such as poly(L-lactic-co-glycolic-acid) (PLGA) polymer foams, collagen hydrogels, and porous alginate material for adipose tissue engineering. While materials such as PLGA foams are too rigid for soft tissue deficit correction, injectable hydrogel materials that are polymerizable *in vivo* are more appropriate for this application. The goal of this research is to initially provide a material to act as a support system for preadipocyte adhesion and growth, with the understanding that it will remodel with time and be replaced eventually with adipose tissue.

It is hypothesized that a photopolymerizable derivatized poly(ethylene glycol) hydrogel system can be a three-dimensional scaffold system that aids in the support and proliferation of preadipocytes. This polymer meets our initial specifications for an ideal scaffold material because it can be modified to incorporate cell-specific degradation sites and adhesion ligands to accommodate cellular adhesion and proliferation, which is crucial to cell function. The preadipocytes can be homogeneously mixed into the

prepolymer solution and then photopolymerized into a hydrogel. Three derivatives of poly(ethylene glycol) were fabricated: diacrylated poly(ethylene glycol) which is nondegradable, poly(ethylene glycol) incorporated with a collagenase-sensitive degradable sequence (LGPA), and poly(ethylene glycol) coupled with cell-adhesion ligands (YIGSR). Four scaffold configurations were prepared and analyzed: nondegradable scaffold, nondegradable scaffold containing cell-adhesion ligands, degradable scaffold, and degradable scaffold containing cell-adhesion ligands. Proliferation and viability assays were completed to determine which scaffold configuration is best suitable for preadipocyte growth.

Next, the proliferative capabilities of preadipocytes within the hydrogels were assessed. DNA assays demonstrated that preadipocytes are anchorage dependent cells. In other words, cell viability and proliferation are dependent on the adhesion to the underlying substratum. One of the first studies performed was to assess the adhesivity of the incorporated ligand. It was observed that preadipocytes were able to adhere to hydrogels containing YIGSR within three hours.

Preadipocytes did not proliferate while in the nondegradable poly(ethylene glycol) scaffold due to the lack of degradation sites. In order for cells to undergo morphological changes during proliferation, there must be adequate room. Nondegradable poly(ethylene glycol) hydrogels do not provide the additional space required for continued cell proliferation. In addition, due to the absence of adhesion sites, cells cannot adhere to the polymer to provide traction for cell division. Moreover, DNA content in nondegradable

hydrogels containing YIGSR remained constant during the course of the study. Cell-adhesion sites allowed the preadipocytes to bind to the hydrogel, but due to the nondegradable nature of the network, the cells are contact inhibited and no longer proliferate.

In contrast, the DNA content in the degradable scaffold increases initially, but subsequently decreases to initial levels. Serum protein from the DMEM used to form the hydrogels permits temporary preadipocyte binding and initial degradation of the network by cellular secretion of collagenase results in initial proliferation. As degradation continues to occur, the serum protein is no longer entrapped, thus cell proliferation decreases. As a result, there is initial cell proliferation followed by cell death, as the hydrogel cannot support continued cell adhesion. Only when poly(ethylene glycol) scaffolds containing both cell-adhesion ligands and degradation sequences does the DNA content increase steadily over the course of the study. Cells are able to attach to the supporting network, proliferate, and secrete collagenase to degrade the polymer. Hence a four-to-five-fold increase in cell growth is observed over the course of the week.

Results of *in vitro* experiments indicate that degradable networks allow for cellular growth while nondegradable networks do not. In addition, incorporating cell-adhesion sites are necessary for supporting cell growth. Results also demonstrate that preadipocyte adhesion and the sustenance of cellular growth depend upon both the degradation and adhesion sites in the system. Combining the degradable poly(ethylene glycol) with the

polymer coupled with cell-adhesion sites produces a synthetic hydrogel scaffold that has been shown to promote preadipocyte proliferation and viability.

Poly(ethylene glycol) modified with LGPA and YIGSR peptide sequences proves to be a suitable biocompatible material for this application. Poly(ethylene glycol) is also relatively cost effective and may ideally provide longer term results than current methods of repairing defects. It is easily injectable and can be polymerized and molded to fill the defect volume *in vivo*, which makes it an ideal material to be used in the clinical setting.

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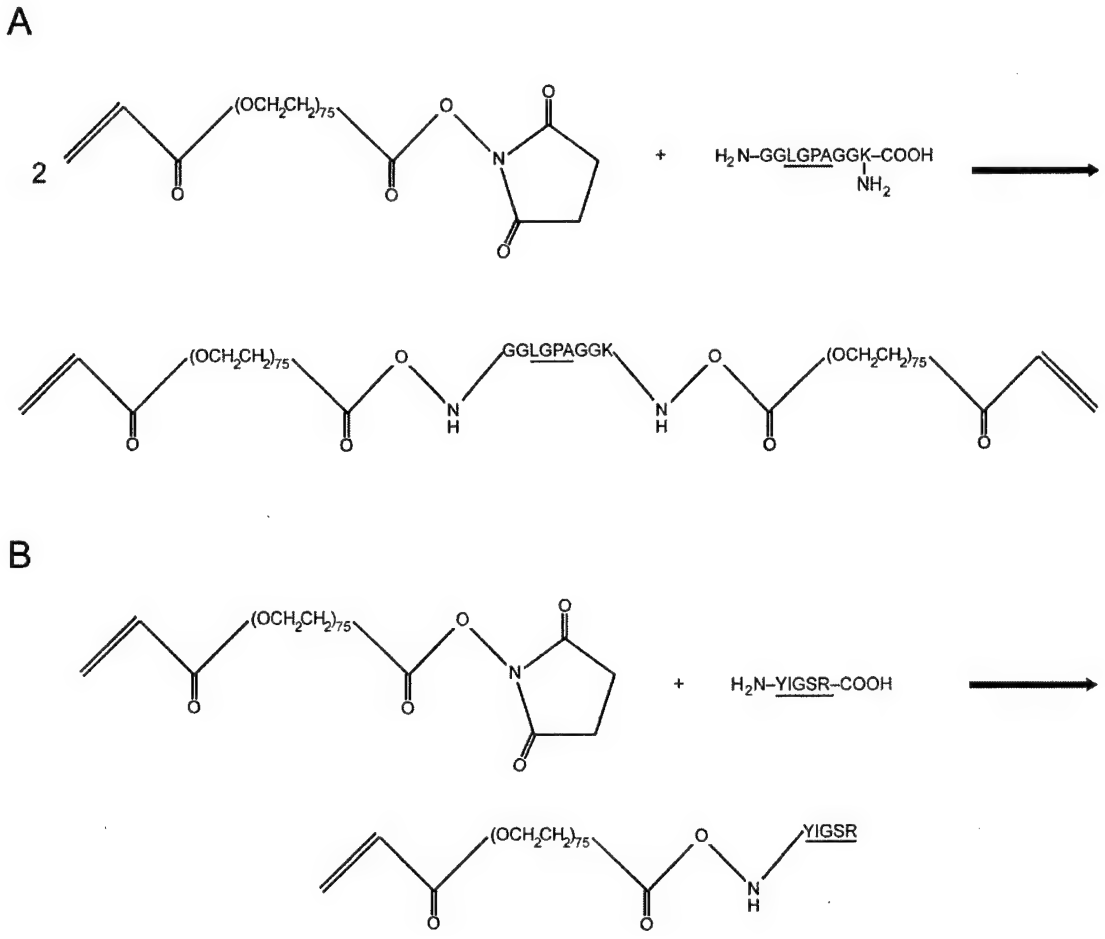

Figure 1. (A) Coupling of collagenase sensitive peptide, LGPA, with acrl-PEG-NHS to form a degradable hydrogel, ABA block copolymer, terminated in acrylate groups for photopolymerization. (B) Coupling of cell adhesive peptide, YIGSR, with acrl-PEG-NHS to facilitate preadipocyte binding to hydrogel.[21]

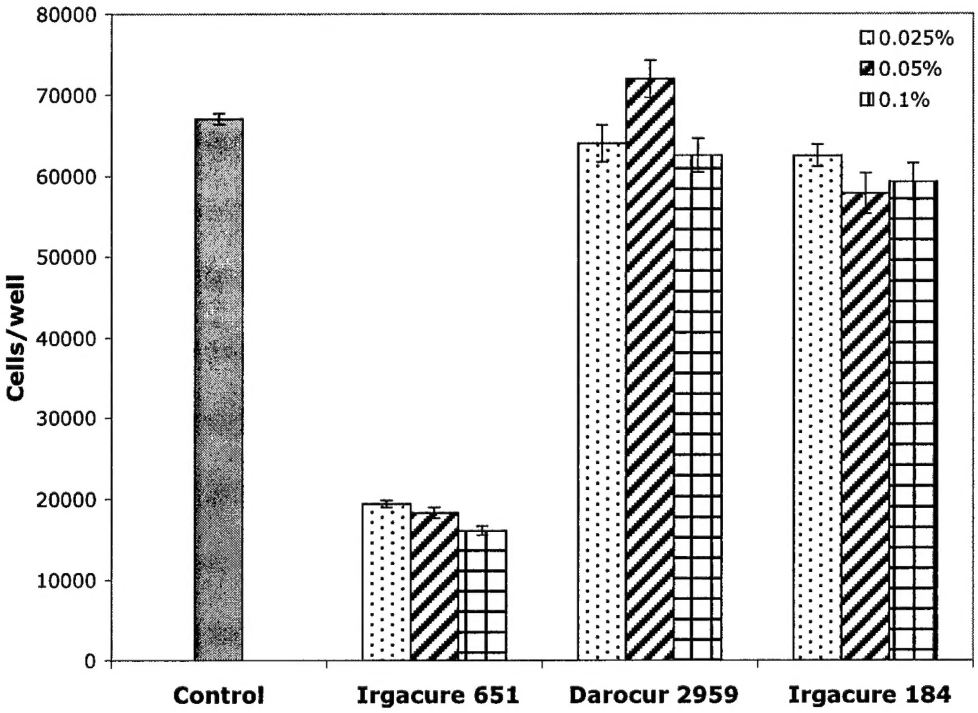

Figure 2. Preadipocyte response to varying concentrations of Irgacure 651, Darocur 2959, and Irgacure 184. Data are average cell count (n=3) ± standard error of the mean (SEM).

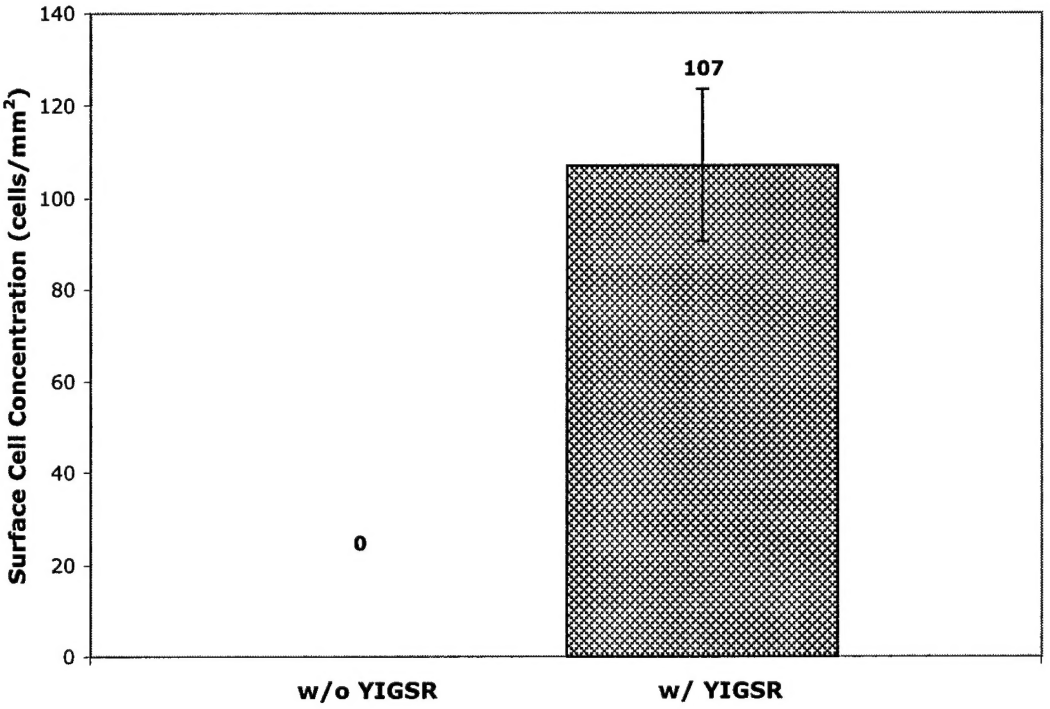

Figure 3. Average concentration of preadipocytes (± SEM) on surface of hydrogels with and without the YIGSR adhesion ligand.

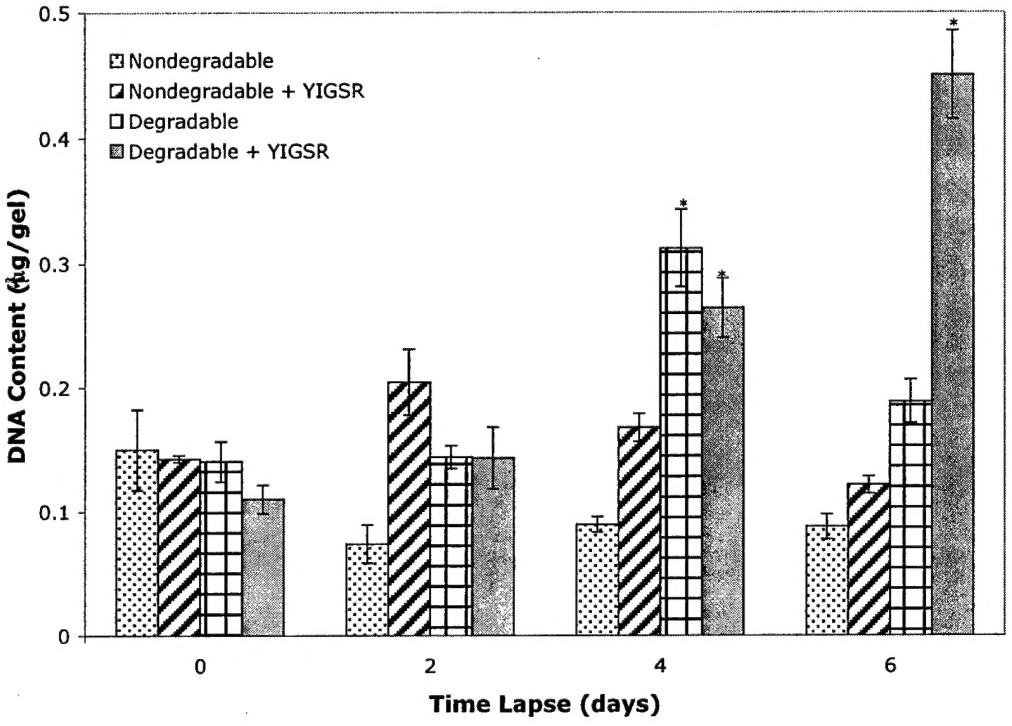

Figure 4. Average DNA content (± SEM) in hydrogels vs. time. (*) denotes statistical significance when compared to day 0.

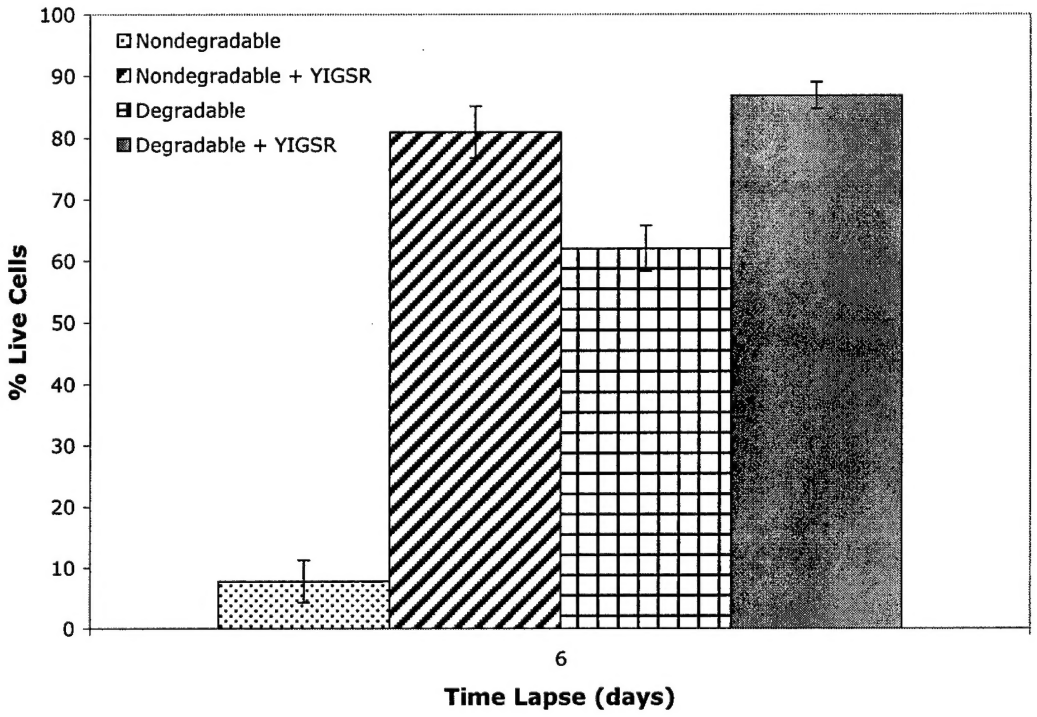

Figure 5. Average percent of viable cells (± SEM) in each hydrogel configuration after six days.